BRIEF REPORT

IDENTIFICATION OF *Leishmania* SPECIES IN PATIENTS DERIVED TO THE NATIONAL INSTITUTE OF HEALTH, PERU

Aidé Sandoval-Juárez [1,a], Gloria Minaya-Gómez [1,b], Nyshon Rojas-Palomino [1,c], Omar Cáceres [2,3,d]

[1] Laboratorio de Referencia Nacional de Leishmaniasis, Instituto Nacional de Salud, Lima, Peru.
[2] Laboratorio de Biotecnología y Biología Molecular, Instituto Nacional de Salud, Lima, Peru.
[3] Escuela de Medicina Humana, Facultad de Ciencias de la Vida, Universidad Científica del Sur, Lima, Peru.
[a] Biologist, master in Public Health; [b] biologist, master in Tropical Medicine and International Health; [c] biologist;
[d] biologist, master in Biochemistry and Molecular Biology.

ABSTRACT

In Peru, leishmaniasis is a metaxenic disease that represents a serious public health problem, due to its wide distribution and the number of people in danger of contracting the disease, being the vulnerable population mainly those with low economic resources. The study was conducted from patients who were derived to Peru's National Institute of Health between 2006 and 2011 so that the specialized diagnosis could be carried out. The identification of the species of infectious Leishmania was developed through the analysis of the High-Resolution Melting Analysis obtained from the genomic DNA of promastigotes and amastigotes, which allows to identify the species of *Leishmania (Viannia) braziliensis, Leishmania (V.) guyanensis, Leishmania (V.) peruviana* as more prevalent, in addition to *Leishmania (V.) lainsoni* and *Leishmania (L.) amazonensis*.

Keywords: Cutaneous Leishmaniasis; kDNA minicircles; Molecular Typing; Real-Time PCR; Peru (source: MeSH NLM).

INTRODUCTION

Leishmaniasis is a neglected vector-borne disease. In the last five years, approximately one million cases of cutaneous leishmaniasis have been reported worldwide, with an estimated 220,000 cases per year and more than 399 million people at risk of infection [1,2]. In Peru, American tegumentary leishmaniasis (ATL) is considered a public health problem because it predominantly affects the poorest population, due to its wide geographical distribution [3] and the diversity of circulating species. The most prevalent species are *Leishmania (Viannia) braziliensis and Leishmania (V.) guyanensis,* mainly in the regions of Madre de Dios, Ucayali and San Martín; *Leishmania (V.) peruviana in Lima, Ancash, La Libertad and Cajamarca; while Leishmania (L.) amazonensis* has been less reported, mainly in the regions of Junin, Amazonas and Ucayali. Other species such as *Leishmania (V.) lainsoni, Leishmania (V.) shawi, Leishmania (V.) panamensis, Leishmania (V.) colombiensis and the hybrid species Leishmania braziliensis/peruviana* have been sporadically reported [4-7].

Between 2000 and 2018, the Peruvian Ministry of Health (MINSA) reported 135,233 confirmed cases, an average of 7,117 cases per year, of which, approximately 94% (6,685 cases) was cutaneous and 6% (432) had the mucocutaneous form. In 2018, the regions of Madre de Dios and Cusco accounted for 25% of the total number of reported cases [3].

The identification of Leishmania species is done by multiple locus sequence typing (MLST), multilocus enzyme electrophoresis (MLEE), the use of monoclonal antibodies, sequencing of specific molecular markers [8,9] and by amplification of multiple markers in the conventional polymerase chain reaction (PCR) [10]. On the other hand, high resolution melting analysis (HRMA) is a molecular method following real-time PCR that allows, through the analysis of DNA dissociation curves, the molecular identification of each species [11], population, as well as single-nucleotide polymorphism (SNP) [12].

Patients suspicious of leishmaniasis are referred, mainly from national hospitals and military and police institutions located in different endemic areas of Peru to the National Health Institute (INS) which is part of the Health Ministry. The INS carries out the confirmatory diagnosis of the disease. In recently evolved skin cases, diagnosis is mainly achieved by parasitological methods, such as direct microscopic examination or smear, which are widely used due to their low cost, and in vitro culture. The indirect immunofluorescence (IFI) and the Montenegro intradermal test (IDRM) are used for the immunoserological diagnosis of the chronic mucocutaneous form of the disease. On the other hand, despite the efficiency of molecular methods such as PCR for the detection of the parasite, their implementation is restricted to specialized centers with adequate equipment, infrastructure and budget, as well as specialized personnel to guarantee the continuity of the service.

The objective of the present study was to identify the infecting Leishmania species in patients referred to the INS from 2006 to 2011, through the analysis of DNA dissociation curves.

THE STUDY

Observational, descriptive and retrospective study. The population studied were patients with clinical suspicion of ATL who attended the INS between 2006 and 2011, referred from national hospitals because they had ulcerous skin lesions with: raised edges, nodules, plaques or that were at mucosal level, The diagnosis was confirmed by parasitological methods at the INS National Leishmaniasis Reference Laboratory.

The identification of *Leishmania* was made from genomic DNA extracted from amastigotes achieved by scraping the skin lesion with a sterile lancet, preserved in 70° alcohol and from promastigotes from in vitro culture. The samples were obtained as part of the development of the diagnostic routine for case confirmation. DNA extraction was performed using the recommendations of the commercial PureLink Genomic DNA kit (Invitrogen, Life-technologies). The DNA samples were eluted in a volume of 60 µl and stored frozen until later use.

The study was performed by amplifying a region of the kinetoplast DNA (kDNA) in a RotorGene Q thermocycler (Qiagen) , in a reaction volume of 10 µl; 2 µl (5 ng/µl) of parasitic DNA were used, as well as 0.7 µM of the A1 primers: 5'-CCG CCC CTA TTT TAC ACC AAC CCC-3 and A2: 5'-GGG GAG GGG CGT TCT GCG AA-3' [13] and 1X of the HRM-PCR Master Mix solution (Tipe-it HRM PCR Kit, Qiagen).

KEY MESSAGES

Motivation for the study: To contribute to the description, updating and geographical distribution of Leishmania species in Peru.

Main findings: The identification of *L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) peruviana*, as species with higher prevalence in the country, in addition to *Leishmania (V.) lainsoni* and *L. (L.) amazonensis,* the latter identified in a patient from Huanuco, an area with no history of previous cases.

Implications: Rapid and timely identification of the infecting Leishmania species by the analysis of the dissociation curves by HRMA, is important to determine the prognosis of the disease and for the development of future studies about differentiating species of Leishmania circulating in Peru.

The identification of *Leishmania* species was developed through the analysis of dissociation curves using reference strains as control samples, the WHO codes are detailed in Annex 1. The HRMA identification of a test sample was achieved by comparing the obtained profile with a control, and was expressed in percentage of similarity by the Rotor Gene Q Series Software version 2.3.1.

Likewise, with the purpose of confirming the cases found in the identification of Leishmania by HRMA, the concordance with 14 samples randomly selected by sequencing of the Leishmania spp, cytochrome B gene was determined according to the methodology described by Foulet *et al.* [8] The primers used were Lei-CytB09: 5'-TTATGGTGTAGGTTTTAG-TYTAGGTT-3' and Lei-CytB12: 5'-TGCTAAAACCACTCA-TAAATATACT-3'. The sequences obtained were analyzed and compared with the GenBank database (https://www.ncbi.nlm.nih.gov/genbank).

The frequency of the variables age, sex, occupation, origin, duration of illness, number of lesions, type of lesions, location and total surface area of the lesion were determined. In addition, using the variables of the probable site of infection (at the regional level) and the identified Leishmania species, a distribution map was developed with the Quantum GIS version 3.8 geographic information system.

The study was approved by the INS Research Ethics Committee. Authorization for the use and analysis of the strains was also requested from the National Reference Laboratory for Leishmaniasis

RESULTS

Between January 2006 and December 2011, 262 patients with clinical suspicion of leishmaniasis were referred to the INS. Only

101 patients met the criteria for the parasitological diagnosis of the disease, 69 (68.3%) of which were confirmed for ATL and 32 (31.7%) were negative in parasitological and immunoserological diagnosis of the disease.

Regarding confirmed patients, by means of HRMA and by comparison with the control samples, the identification of Leishmania was achieved in 45 patients; while, in the remaining 24 it was not possible to determine the species of infecting *Leishmania*, due to the similarity found against the controls below the established cut-off value.

From the 45 patient samples confirmed by HRMA, 40% were identified as *Leishmania (V.)* braziliensis, 28.9% as *Leishmania (V.) guyanensis,* 17.8% as *Leishmania (V.) peruviana,* 6.7% as *Leishmania (L.) amazonensis,* and 6.7% as *Leishmania (V.) lainsoni* (Figure 1).

For the identification of the species, a similarity was assumed to be ≥98% as the cut-off value, with the exception of the samples identified as *Leishmania (V.) lainsoni* which cut-off value was ≥78%. With this criterion, the infecting *Leishmania s*pecies was identified in a total of 45 patients, 42 of which presented a similarity of ≥98% and the remaining three a similarity of ≥78%.

From the 45 patients identified, 43 (96%) presented the cutaneous form, of which 17 (38%) were caused by *Leishmania (V.) braziliensis* from Amazonas, Cajamarca, Cusco, Huanuco, Loreto, Madre de Dios and San Martin; 12 (27%) for *Leishmania (V.) guyanensis* from Amazonas, Cusco, Huanuco, La Libertad, Lima and San Martin 8 (18%) for *Leishmania (V.) peruviana* from Amazonas, Ayacucho, Huanuco, Junin, Lambayeque and Lima; 3 (7%) *for Leishmania (V.))* lainsoni from the provinces of La Convencion, Satipo and Huaura in the regions of Cusco, Junin and Lima, respectively; and 3 (7.0%) for *Leishmania (L.) amazonensis* from the provinces of La Mar and Huanta in the region of Ayacucho and Churubamba in Huanuco (Annex 2).

In the latter locality, the described species was identified from a patient with the disseminated cutaneous form. Finally, 4% of the population were two patients from the provinces of Puerto Inca and Huanuco affected by the mucocutaneous form, in whom we were able to identify *Leishmania (V.) braziliensis* and *Leishmania (V.) guyanensis*, respectively.

Likewise, the results identified by HRMA and the 14 randomly sequenced samples determined a 100% concordance.

Figure 1. Lesions caused by **(A)** *Leishmania (V.) guyanensis,* **(B)** *Leishmania (V.) braziliensis,* **(C)** *Leishmania (L.) amazonensis,* **(D)** *Leishmania (V.) lainsoni,* and **(E)** *Leishmania (V.) peruviana*

The clinical-epidemiological characteristics of the 45 patients, in whom the identification of the infecting Leishmania species was achieved are detailed in Table 1.

DISCUSSION

The identification of Leishmania was conducted in 45 patients confirmed for ATL through the analysis of the kDNA dissociation curves, achieving identification of Leishmania *(V.) braziliensis, Leishmania (V.)* guyanensis, *Leishmania (V.) peruviana, Leishmania (V.) lainsoni* and *Leishmania (L.)* amazonensis, species reported in previous studies [4,14].

For the identification of the Leishmania species, a cut-off value was established related to the degree of genetic divergence, the close relationship between *Leishmania (L.)* amazonensis and *Leishmania (L.)* mexicana in the subgenus *Leishmania* and *Leishmania (V.)* braziliensis, Leishmania (V.) peruviana, *Leishmania (V.) guyanensis* and *Leishmania (V.) panamensis* in the subgenus *Viannia* has led to the selection of the ≥98% similarity as the cut-off value, unlike the ≥78% similarity assumed for *Leishmania (V.) lainsoni,* because this species presents particular characteristics at the kDNA level, which differs from the other species in the subgenus *Viannia* [15,16]. In our study, this species was identified in samples from three patients from Cusco, Junin and Lima. In the latter, confirmation was achieved by sequencing the cytochrome B gene, while the samples from Cusco and Junin were confirmed by polymorphic length restriction fragment analysis (PCR-RFLP) [17]. Conversely, samples from patients who did not reach the cut-off value were not considered because identification might not be correct.

Likewise, from the 45 samples identified, 61% were isolated from patients from the jungle region and 39% from patients from the sierra, mainly from the western slopes of the Andes and inter-Andean valleys, which allowed us to determine *Leishmania (V.) braziliensis* followed by *Leishmania (V.) guyanensis* and *Leishmania (V.) peruviana* as the most prevalent species, widely reported in our country [7,18]. However, their ordinal position may vary depending on the origin of the population, as evidenced in the study conducted by Lucas *et al.* in which *Leishmania (V.) braziliensis* was most frequent in the jungle region, followed by *Leishmania (V.) peruviana* and *Leishmania (V.) guyanensis.*

Similarly, studies by Arevalo *et al.* and Kato *et al.* in predominantly sierra populations, where *Leishmania (V.)* peruviana mainly circulates, determined this species as the most prevalent, followed by *Leishmania (V.) braziliensis* and *Leishmania (V.) guyanensis.* Other species reported from the western slopes of the Andes and inter-Andean valleys were *Leishmania*

Table 1. Sociodemographic and epidemiological characteristics of confirmed cases of leishmaniasis, 2006-2011 (n=45)

Demographic Variables	n (%)
Age (years)	
0-4	5 (11.1)
5-16	7 (15.6)
17-26	10 (22.2)
27-64	18 (40)
>65	5 (11.1)
Sex	
Female	18 (40)
Male	27 (60)
Employment	
Agriculture	17 (37.8)
Mining	1 (2.2)
Students	8 (17.8)
Police and military	4 (8.9)
Health professionals	2 (4.4)
Intradomiciliary activity (housewives, etc.)	8 (17.8)
Others [a]	5 (11.1)
Origin	
Sierra	18 (40.0)
Jungle	27 (60.0)
Clinical Variables	
Clinical type	
Cutaneous [b]	43 (95.6)
Mucocutaneous	2 (4.4)
Duration of disease (months)	
<1	5 (11.1)
1-2	19 (42.2)
3-6	15 (33.3)
>6	6 (13.3)
Number of lesions	
1	31 (68.9)
2	5 (11.1)
≥3	9 (20.0)
Lesion type	
Ulcer	40 (88.9)
Ulcer and nodules	2 (4.4)
Only nodules	3 (6.7)
Lesion location	
Head [c]	16 (35.6)
Torso [d]	1 (2.2)
Upper or lower limbs [e]	28 (62.2)
Compromised area (mm)	
<10	8 (17.8)
11-20	17 (37.8)
21-30	10 (22.2)
>30	10 (22.2)

[a] Merchant, bricklayer, engineer, teacher, civil employee, builder; [b] data includes a case of disseminated cutaneous leishmaniasis; [c] nostrils, lips, ears, cheeks, forehead, chin, head, neck; [d] chest, back, hips; [e] leg, calf, foot, knee, ankle, thigh, hand, finger, arm, forearm, elbow

(V.) braziliensis in Ancash and Lima [18], and *Leishmania (V.) guyanensis* in Lambayeque, Lima [19] and La Libertad [14].

With respect to the mucocutaneous type, parasites obtained from two patients from Huanuco, *Leishmania (V.) braziliensis* and *Leishmania (V.) guyanensis* were identified as the infecting species, both with recognized capacity to develop metastasis and to evolve into aggressive forms of the disease [20,21]. For the clinical mucocutaneous form, between 5% and 20% of total cases of leishmaniasis have been reported, depending on the geographical region [21]. In Peru, approximately 6% of the mucocutaneous form of the total annual cases is reported [3]. *Leishmania (L.)* amazonensis was also identified as the causal agent of disseminated cutaneous leishmaniasis in a patient from Huanuco. Cases of leishmaniasis with this clinically disseminated form are unusual and are generally investigated as case reports.

The 100% concordance obtained between the Sanger method sequencing and HRMA allows us to reaffirm the capacity of the latter, and of kDNA used as a marker in the identification of the infecting Leishmania species and, unlike sequencing, with less consumption of time and economic resources.

The limitations of the present study are related to the period in which the samples were taken (2006-2011), to the unequal geographical distribution of the patients, predominantly from the jungle region, to the lack of identification of the total of confirmed samples due to the low parasitic load which did not made possible to reach the cut-off value established for identification by HRMA, to the high costs of identification by sequencing using the Sanger method. It is because to these limitations that it was not possible to confirm the remaining 31 identified samples.

In conclusion, from the analysis of the DNA dissociation curves or HRMA of 45 patients, we were able to identify the species of *Leishmania (V.) guyanensis, Leishmania (V.) peruviana, Leishmania (V.) braziliensis, Leishmania (V.)* lainsoni and *Leishmania (L.) amazonensis* circulating in 13 regions of Peru with indigenous transmission of leishmaniasis. Likewise, we reported for the first time the circulation of *Leishmania (V.) amazonensis* in the Huanuco region, identified from a case with disseminated cutaneous leishmaniasis. Continuing studies related to the identification of Leishmania species with a greater number of samples will allow us to have a better knowledge of the distribution and dispersion of *Leishmania spp* species, considering the displacement of the vector, climate variations, migrations, mining activities, among other factors.

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